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Bayesian analyses of admixture in wild and domestic cats (*Felis silvestris*) using linked microsatellite loci

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Abstract

Methods recently developed to infer population structure and admixture mostly use individual genotypes described by unlinked neutral markers. However, Hardy–Weinberg and linkage disequilibria among independent markers decline rapidly with admixture time, and the admixture signals could be lost in a few generations. In this study, we aimed to describe genetic admixture in 182 European wild and domestic cats (*Felis silvestris*), which hybridize sporadically in Italy and extensively in Hungary. Cats were genotyped at 27 microsatellites, including 21 linked loci mapping on five distinct feline linkage groups. Genotypes were analysed with STRUCTURE 2.1, a Bayesian procedure designed to model admixture linkage disequilibrium, which promises to assess efficiently older admixture events using tightly linked markers. Results showed that domestic and wild cats sampled in Italy were split into two distinct clusters with average proportions of membership $Q > 0.90$, congruent with prior morphological identifications. In contrast, free-living cats sampled in Hungary were assigned partly to the domestic and the wild cat clusters, with $Q < 0.50$. Admixture analyses of individual genotypes identified, respectively, 5/61 (8%), and 16–20/65 (25–31%) hybrids among the Italian wildcats and Hungarian free-living cats. Similar results were obtained in the past using unlinked loci, although the new linked markers identified additional admixed wildcats in Italy. Linkage analyses confirm that hybridization is limited in Italian, but widespread in Hungarian wildcats, a population that is threatened by cross-breeding with free-ranging domestic cats. The total panel of 27 loci performed better than the linked loci alone in the identification of domestic and known hybrid cats, suggesting that a large number of linked plus unlinked markers can improve the results of admixture analyses. Inferred recombination events led to identify the population of origin of chromosomal segments, suggesting that admixture mapping experiments can be designed also in wild populations.

Keywords: Bayesian admixture analysis, conservation genetics, *Felis silvestris*, hybridization, linked microsatellites, wild and domestic cat

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Introduction

A number of procedures aimed to infer cryptic population structure and admixture have been recently developed (Pritchard *et al*. 2000; Dawson & Belkhir 2001; Corander *et al*. 2003). These methods use genotypes defined by multiple unlinked neutral markers to model allele frequencies in source populations, which do not need to be explicitly identified, and to assign individuals to one or more than one population. Population structure is modelled assuming that admixture led to transient Hardy–Weinberg and linkage disequilibria (HWD and LD; Pritchard *et al*. 2000). However, genetic disequilibrium among unlinked markers are expected to decline rapidly, leading the admixture

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signals to fade in a few generations (Falush *et al*. 2003). A new Bayesian procedure (the 'linkage model', implemented in the software STRUCTURE 2.1; Falush *et al*. 2003), which has been designed to model the correlations between linked loci arising in admixed populations ('admixture LD'), is aimed to detect more ancient admixture events using tightly linked markers. STRUCTURE 2.1 models linkage decay as a Poisson process of rate 1 per Morgan and computes the parameter *r*, an estimate of the number of generations since the admixture. The correlations among linked loci should allow to obtain more accurate estimates of admixture, to identify recombination events, and eventually to infer the population of origin of chromosomal segments. Moreover, STRUCTURE 2.1 introduces a new model for correlated allele frequencies among populations which diverged recently, or that are connected by gene flow. This model promises to be efficient in identifying shallow population structure (Falush *et al*. 2003). Linked markers are frequently used in human population genetics and animal model species to estimate linkage disequilibria, admixture proportions and in admixture mapping of candidate genes (Seldin *et al*. 2004), but they have not been applied in natural animal populations. In this study we used, for the first time, a panel of linked microsatellite markers to genotype wild and domestic cats, which are known to hybridize extensively in parts of their distribution range (Beaumont *et al*. 2001; Randi *et al*. 2001; Pierpaoli *et al*. 2003).

The European (*Felis silvestris silvestris*), African (*Felis silvestris libyca*) and domestic (*Felis silvestris catus*) cats are distinct subspecies of a single polytypic species, *Felis silvestris* (Ragni & Randi 1986; Randi & Ragni 1991; Wozencraft 1993; Johnson & O'Brien 1997; Randi *et al*. 2001). The endangered European wildcat is nowadays distributed in fragmented populations ranging from Russia to Portugal, and from Scotland to the Near East (Nowell & Jackson 1996). The post-Pleistocene historical range of the wildcat was much wider, perhaps including all the forested areas in Europe and mainland Britain, but during the 18th and 19th centuries wildcat populations declined, were locally eradicated, and went fragmented due to deforestation, trapping and hunting. Concomitantly, the anthropogenic diffusion of domestic cats throughout Europe originated widespread free-ranging populations in agricultural ecosystems, raising concerns about the genetic integrity of wildcats, which could have been hybridizing with domestic cats for centuries (McOrist & Kitchener 1994). Indeed, Suminski (1962), Mendelssohn (1999) and Stuart & Stuart (1991) suggested that hybridization might have led to the genetic extinction of local wildcat populations in central Europe, the Near East and South Africa. The domestication process did not deeply change body size and shape of domestic cats (except for the obvious coat colour mutations that were recently selected by cat breeders), which made it difficult to identify 'pure' wildcat specimens to be used as reference in hybridization studies (Daniels *et al*. 1998).

Limited distinction between wild and domestic cats was found using allozyme electrophoresis, DNA analyses of nuclear and mitochondrial genes (Randi & Ragni 1991; Hubbard *et al*. 1992), while microsatellite loci provided more clear results (Beaumont *et al*. 2001; Randi *et al*. 2001; Pierpaoli *et al*. 2003). Bayesian clustering and admixture analyses (Pritchard *et al*. 2000) of multilocus genotypes have been used to evaluate the genetic structure of wildcat populations, and assess the extent of hybridization in Scotland (Beaumont *et al*. 2001), in Italy (Randi *et al*. 2001) and elsewhere in Europe (Pierpaoli *et al*. 2003). In this study, we analysed allele frequency variation in wild and domestic cat populations at 27 microsatellites, including 21 linked loci belonging to five linkage groups mapping on distinct chromosomes (Menotti-Raymond *et al*. 1999, 2003). We aimed to compare two populations of wild-living cats, which were previously studied using unlinked microsatellites: the first one, sampled in Hungary, showed widespread hybridization with domestic cats, while the other, sampled in Italy, was sharply distinct from domestic cats and showed very few hybrids (Pierpaoli *et al*. 2003). In this study, we added new cat samples, which were genotyped using both unlinked and linked microsatellites, and that were analysed using the Bayesian linkage model implemented in STRUCTURE 2.1. Results are evaluated aiming to assess if linked multilocus genotypes can improve the power of individual admixture analyses as tools in population genetics studies and conservation of wildcats in Europe.

Materials and methods

Sampling and DNA analyses

We collected a total of 182 tissue or blood samples (stored, respectively, in 95% ethanol or in a Tris/SDS buffer, and kept at –20 °C) from 65 free-living cats from Hungary, 61 putative wildcats from Italy, 50 domestic cats (sampled through veterinary practices in Italy, $n = 40$, and Germany, $n = 10$), and 6 known European wild × domestic cat captive-reared hybrids (two F_1 and four backcrosses with wildcats reared in Italy), which were included as a reference. Free-living cats sampled in Italy included road kills, shot or trapped animals from central Apennines, Tuscany and Sicily (Fig. 1a), which were a priori identified as putative European wildcats, domestic cats or hybrids according to coat-colour patterns (Ragni & Possenti 1996), cranial and/or intestine indices (Schauenberg 1977a, b; Ragni 1981), independently of any genetic information (see Pierpaoli *et al*. 2003). The free-living cats that were collected in 10 localities in Hungary (Fig. 1b) showed extensive morphological variation (ranging from putative 'pure wild' to 'domestic' coat-colour patterns), but were pooled in a single group that was difficult to split

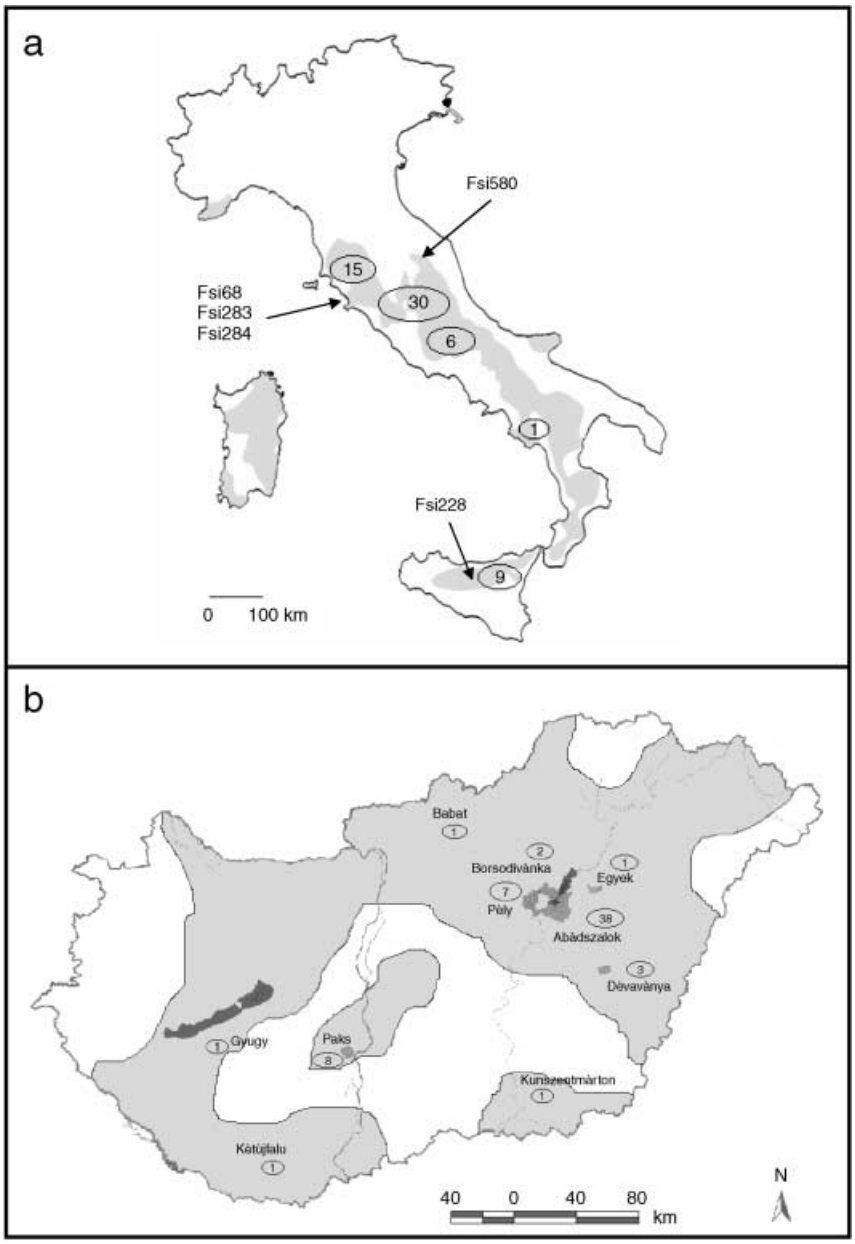

Fig. 1 Approximate ranges of wildcat distributions in Italy (a) and Hungary (b). Sampling locations and size are indicated. Arrows indicate the locations of admixed wildcats in Italy.

due to evidence of widespread hybridization and deep introgression (Pierpaoli *et al*. 2003).

Total DNA was extracted using guanidine thiocyanate (Gerloff *et al*. 1995). Polymerase chain reaction (PCR) amplifications of individual microsatellites were performed either following Menotti-Raymond *et al*. (1999), or as follows: first denaturation at 94 °C for 2 min, 30 cycles of denaturation at 94 °C (15 s), annealing at 54–60 °C (15 s) and extension at 72 °C (30 s), final extension at 72 °C for 10 min. PCRs were performed in 10-μL volumes containing 1 × *Taq* buffer, 0.5 μM each of forward and reverse primers, 2 mM $MgCl_2$, 0.4 mM dNTPs, 0.01% BSA, 0.4 U *Taq* DNA polymerase, and 20–40 ng of genomic DNA. Diluted PCR products were pooled in sets of three to five loci and analysed using an ABI 3100 automated sequencer with the software GENOTYPER 2.1.

We genotyped 21 linked microsatellites, mapping in the domestic cat genetic linkage groups A1, A3, B1, C2 and D3 (Menotti-Raymond *et al*. 2003), and 6 unlinked microsatellites (Table 1). Distances between linked loci ranged from 1 cM (between FCA153 and FCA229; FCA161 and FCA171) up to 86 cM (between FCA23 and FCA126). A few markers overlapping on the same map positions in the genetic linkage groups (Menotti-Raymond *et al*. 2003; their Fig. 1) were considered distant 0.5 cM (Table 1). Data were analysed using separately the total set of 27 loci, or the subset of 21

Table 1 Summary of allelic variation at 21 linked and 6 unlinked feline microsatellite loci used to genotype wild and domestic cats (*Felis silvestris*). Microsatellite IDs, linkage groups and map distances are indicated according to Menotti-Raymond *et al*. (2003). Intermarker distances start from the first locus of each linkage group, which is indicated by a distance value of 0 cM. A distance of 0.5 cM was added to loci which overlap in the same position of the domestic cat genetic linkage map (Menotti-Raymond *et al*. 2003; their Fig. 1). Deviations from Hardy–Weinberg equilibrium were estimated from F_{IS} for each locus and each cat group. Significant F_{IS} values (at a probability level equivalent to $P < 0.05$ after Bonferroni correction for multiple comparisons) are indicated by an*

Microsatellite ID	Linkage groups (distances in cM)	Allele numbers	Allele size range	F_{IS} Domestic	F_{IS} Italy wild	F_{IS} Hungary
Linked loci						
FCA24	A1 (0)	11	214–234	0.047	0.135	0.138
FCA571	A1 (0.5)	10	102–120	0.223	0.147	0.195*
F146	A1 (0.5)	7	139–157	0.092	0.026	–0.018
FCA153	A1 (7)	10	128–150	0.022	–0.027	–0.039
FCA229	A1 (1)	13	144–170	–0.037	0.243	0.062*
FCA161	A3 (0)	7	176–188	0.159	0.232	–0.059
FCA171	A3 (1)	10	98–118	–0.123	0.040	0.092
F85	B1 (0)	43	213–325	0.078	0.012	0.030
FCA577	B1 (2)	12	202–226	0.250*	0.072	0.244
FCA522	B1 (5)	17	125–159	0.050	0.057	0.283
FCA23	B1 (35)	13	127–151	0.148	0.192	0.096
FCA126	B1 (86)	11	109–147	0.044	–0.082	–0.007
F164	C2 (0)	7	146–170	0.046	0.305	0.052
FCA547	C2 (4)	11	231–253	0.245*	–0.025	0.104
FCA576	C2 (0.5)	11	129–149	0.108	0.143	0.111
FCA77	C2 (2)	10	137–159	0.061	0.276*	0.080
FCA43	C2 (74)	10	114–132	0.025	–0.015	0.161
FCA675	D3 (0)	14	195–223	0.129	0.026	–0.103
FCA66	D3 (2)	12	128–154	0.590*	0.191	0.083*
FCA132	D3 (2)	18	132–156	0.035	0.037	0.015
FCA26	D3 (22)	14	122–156	0.045	0.108	0.007
Unlinked loci						
Fca8	A1	14	114–146	0.135	0.067	0.025
FCA149	B1	10	116–134	0.182	0.204	0.134
FCA88	B3	12	101–123	0.092	0.334	0.111
FCA45	D4	19	137–156	0.195*	0.244*	0.093
FCA58	E2	9	213–229	0.087	0.155	0.116
FCA96	E2	19	177–229	0.021	0.055	0.096
Average F_{IS}				0.109	0.112	0.078
(90% CI)				(0.035–0.141)	(0.066–0.140)	(0.044–0.098)
Average H_O				0.701	0.642	0.749
(SE)				(0.125)	(0.147)	(0.087)
Average H_E				0.778	0.716	0.805
(SE)				(0.083)	(0.122)	(0.051)

linked loci, and the unlinked or linkage models as implemented in STRUCTURE 2.1 (Falush *et al*. 2003).

Analyses of genetic variation

Allele frequencies, single and multilocus observed (H_O) and expected (H_E) heterozygosities, deviations from Hardy–Weinberg (HWE) and linkage (LE) equilibria [using Weir's *R* (1979)], and *F*-statistics were evaluated in the complete data set, and for each group (i.e. domestic cats, Italian wildcats and Hungarian free-living cats) separately using GENETIX 4.02 (Belkhir *et al*. 2001; www.univmontp2.fr/~genetix/genetix.htm). Deviations from HWE were computed from F_{IS}, and were tested for both heterozygote deficit and excess using a Markov chain method implemented in GENEPOP 3.2 (Raymond & Rousset 1995; http://wbiomed.curtin.edu.au/genepop/genepop_op1.html). We used default values in GENEPOP, except the number of batches that was increased to 500 to keep the standard errors < 0.01. Bonferroni corrections were applied in multiple comparisons (Rice 1989). GENETIX was also used to describe population differentiation by factorial correspondence analysis (CA; Benzécri 1973) of individual multilocus genotypes.

Bayesian clustering and admixture analyses

We used STRUCTURE (Pritchard *et al*. 2000; http://pritch.bsd.uchicago.edu) to infer the number of *K* unknown populations (genetic clusters) in which the sampled multilocus genotypes can be split. This model-based Bayesian procedure simultaneously estimates the allele frequencies at each locus in each population, and assigns probabilistically the individuals to the population of origin, or to more than one population, if they are admixed. An updated version, STRUCTURE 2.1 (Falush *et al*. 2003), allows choices to be made among five nonexclusive models: (i) the 'no-admixture model', in which each individual may belong to only one of the inferred clusters; (ii) the 'admixture model', in which admixed individuals may have ancestry in two or more parental populations; (iii) the 'independent frequency model *I*' assumes that allele frequencies in each population evolve independently; (iv) however, for a limited number of generations following a population subdivision, or in consequence of ongoing migration, allele frequencies may be correlated (the '*F* model'); (v) individual genotypes are usually described by a set of unlinked neutral markers, and the inferred populations are in, or as close as possible to genetic equilibrium (HWE and LE); however, the new 'linkage model' allows analysing systems of linked markers, thus accounting for the amount of linkage disequilibrium that arises by admixture ('admixture linkage disequilibrium'; Stephens *et al*. 1994).

In this study, we used STRUCTURE 2.1 with the admixture and the linkage models to analyse the total sample set ($n = 182$) using the linked (21 loci) or the total (27 loci) marker sets, and comparing the results obtained with the independent frequency (*I*) and the *F* models. In each case STRUCTURE was run with five repetitions of 10^5 iterations following a burn-in period of 10^4 iterations, and with values of *K* from 1 to 10. We selected the values of *K* showing the optimal subdivision of the data, using the formula: $[\text{Ln P(D)}_k - \text{Ln P(D)}_{k-1}]$ (Garnier *et al*. 2004), where Ln P(D) is the estimated posterior probability of the data conditional to *K*. For each selected value of *K*, we assessed the average coefficients of membership (*Q*) of the sampled populations (domestic cats, Italian wildcats, Hungarian cats) to the inferred clusters. Then, comparing results from the *I* and *F* models, we assigned each individual to the inferred clusters, using a threshold $q_i > 0.80$ for the assignment of individual genomes to one cluster, or, in the case of admixed individuals, jointly to two or more clusters, if the proportion of membership to each one was $q_i < 0.80$. In this way, we used STRUCTURE to estimate the posterior probability for each individual belonging to one parental population, or having fractions of its genome originating from two or more populations.

The posterior probability of each allele to originate in each of the parental population was estimated in the Italian wildcat and Hungarian free-ranging cat samples separately using STRUCTURE with the linkage and *F* models (very similar results were obtained using the *I* model), with $K = 2$, the SITEBYSITE option active and gametic phase unknown (PHASED = 0). STRUCTURE produced the joint assignment probabilities M1P1 that, for a given locus in a given individual, both M (maternal) and P (paternal) alleles are from population 1, and M2P2 that M and P alleles come from population 2. Admixed genotypes are expressed as M1P2 and M2P1. With no information on the gametic phase, the assignment probabilities for the two classes of recombinant genotypes are expected to be equal. Results are expressed as the Ln of the probability ratio of two likelihood values Ln P(R) (Seldin *et al*. 2004). Values of Ln P(R) > 0 would support the hypothesis in the numerator of the ratio, while values of Ln P(R) < 0 would support the hypothesis in the denominator.

Results

Genetic variation and population diversity

All loci were polymorphic in the 182 genotyped wild and domestic cats (Table 1), showing between 7 and 19 alleles per locus, with the exception of locus F85, which yielded a total of 43 different alleles (already found in Menotti-Raymond *et al*. 1999). Observed heterozygosity was lower than expected in the three cat groups, on average, and most of the F_{IS} values were positive (Table 1). Assuming that each locus is an independent replication of an HWE test in each of the three cat groups, a Bonferroni correction led to a significance level of $P = 0.05/27 = 0.0018$, at which there was a total of nine significant F_{IS} values (over 81 comparisons), distributed almost evenly among sample groups (i.e. there were, respectively, four, three and two significant F_{IS} values in domestic, Hungarian free-ranging and Italian wild cats; Table 1). The average F_{IS} values across loci, however, were not significant. The three cat groups resulted in HWE also using the Markov chain method implemented in GENEPOP.

Linkage disequilibrium was estimated in a total of 1053 locus combinations, that is 720 (68%) combinations among the unlinked loci and 333 (32%) within the linkage groups, which showed, respectively, 66/720 = 9%, and 22/333 = 7% significant Weir's *R* values ($P < 0.05$; without Bonferroni correction). The number of significant *R* values (without Bonferroni correction) was higher than expected by chance (5% = 18 values) in each cat group. Moreover, the patterns of LD were different in the three cat groups, the Hungarian cats showing a higher proportion (35%) of significant *R* values at linked loci than the domestic (11%) and the Italian wildcats (14%). After Bonferroni correction, there were, respectively, one, three and six significant *R* values ($P = 0.05/351 = 0.0001$) in the domestic cats, Italian wildcats and

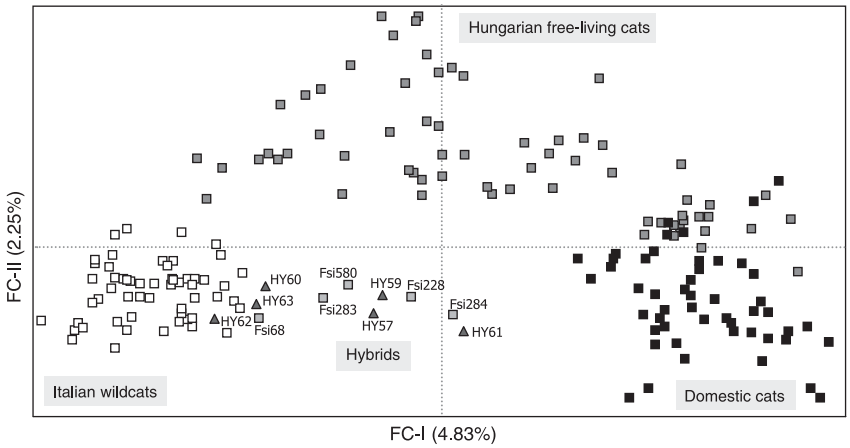

Fig. 2 Factorial component analysis of individual cat genotypes, computed by GENETIX using 27 microsatellite loci. FC-I and FC-II are the first two factorial components. Known hybrids obtained in captivity (HY) and admixed Italian wildcats (Fsi) are indicated.

Hungarian free-living cats. Once again these significant values were mainly among unlinked loci (8/10 cases). Thus, although not pervasive, LD was higher at linked loci in the Hungarian free-living cats relative to the other two groups.

Microsatellite diversity was significantly partitioned between the Italian wild and the domestic cats [Weir & Cockerham's (1984) multilocus $F_{ST} = 0.14$, $P < 0.01$, using either 21 or 27 loci], while F_{ST} was much lower between the Hungarian free-living and the domestic cats ($F_{ST} = 0.04$), or the Italian wildcats ($F_{ST} = 0.08$). The individual genotypes of Italian wild and domestic cats grouped separately on the left and right side of a CA plotting (Fig. 2). In contrast, the Hungarian free-living cats plotted intermediately, overlapping both the domestic and the wild cat distributions, and did not show any obvious splitting of separate subgroups. Five of the six known hybrids (indicated by triangles in Fig. 2) plotted intermediately between the Italian wild and the domestic cats. The hybrid cat HY62 was included within the Italian wildcat group. Five presumed Italian wildcats (Fsi68, Fsi228, Fsi283, Fsi284 and Fsi580) plotted also intermediately, suggesting that they might be hybrids. Similar CA plottings were obtained using 27 loci or only 21 linked loci (not shown).

Assessing cat population clustering by Bayesian analyses

The posterior probability of the data was estimated using STRUCTURE and the total sample set for K from 1 to 10, without any prior population information, with 21 or 27 loci, and the *F* or *I* allele frequency models. Results showed that the values of Ln P(D) increased sharply with K from 1 to 3, then they reached a plateau (Fig. 3). The formula $[\text{Ln P(D)}_k - \text{Ln P(D)}_{k-1}]$ indicated that splitting the samples in three clusters should represent the optimal subdivision of the data, thus avoiding unjustified oversplitting (Fig. 3). Both average and individual assignment probabilities were not affected by using more than three clusters.

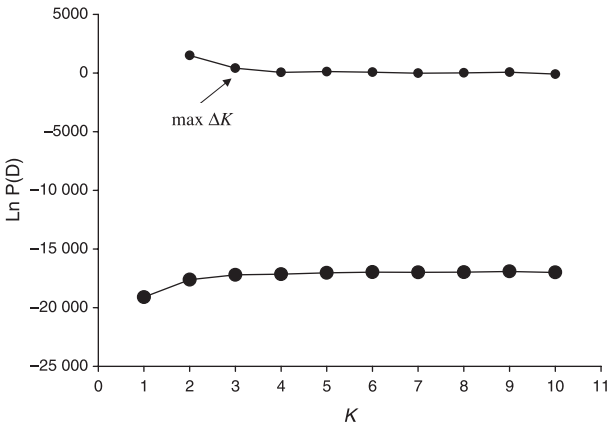

Fig. 3 Posterior probability of the data Ln P(D) against the number of K clusters (below), and increase of Ln P(D) given K, calculated as $[\text{Ln P(D)}_k - \text{Ln P(D)}_{k-1}]$ (above).

Using $K = 3$ and 27 loci, the domestic cats grouped in cluster I with proportion of membership $Q_I = 0.96$, while the Italian wildcats fall into cluster II (with $Q_{II} = 0.91$), concordantly with prior nongenetic classification. The Hungarian wild-living cats were split between the domestic cat cluster I ($Q_I = 0.40$), and clusters II ($Q_{II} = 0.10$) and III ($Q_{III} = 0.50$). These last two clusters represent two distinct genetic populations, respectively, the Italian wildcats (cluster II), and part of the Hungarian (cluster III) free-living cats (see also Fig. 5). The same subdivision, with very similar values of Q, was obtained using the 21 linked markers and either the *I* or *F* models (not shown). These findings indicate that the Italian wildcats are genetically distinct from the domestic cats, while the free-living cats from Hungary include a variety of genotypes ranging from wild and domestic cats to admixed individuals. The known hybrid cats were also split between the wildcat ($Q_{II} + Q_{III} = 0.57$) and the domestic cat clusters ($Q_I = 0.43$), according to their admixed ancestry.

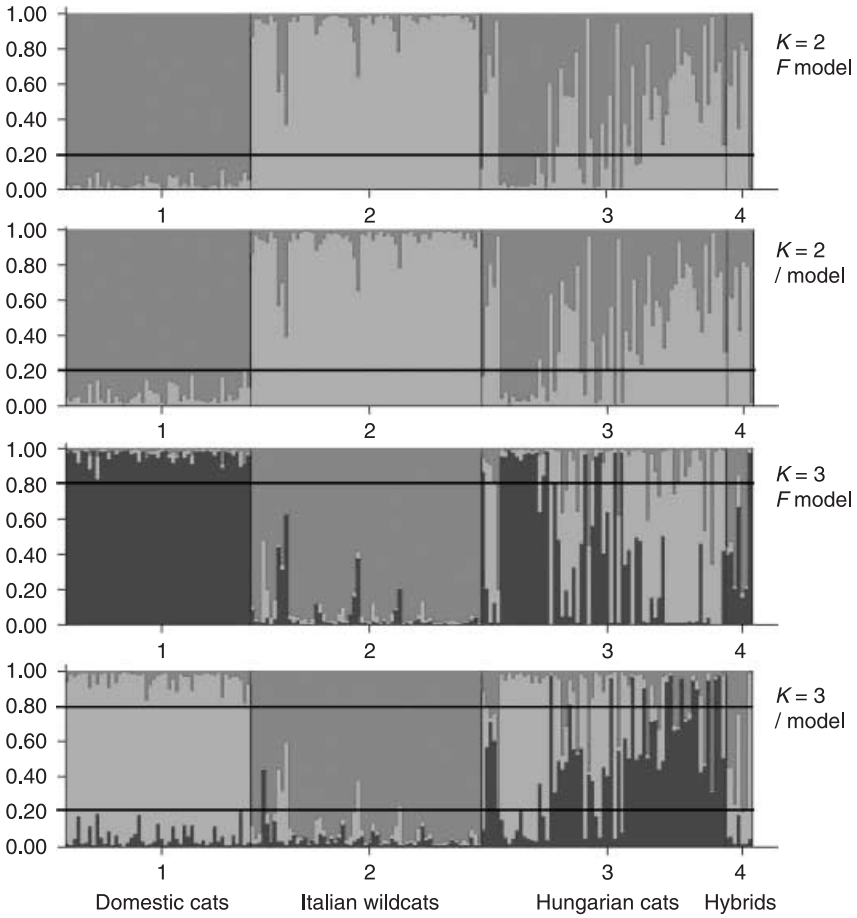

Fig. 4 Admixture analyses of domestic, Italian wild, Hungarian free-living and known hybrid cats (labelled below the plots), performed using STRUCTURE with $K = 2$ or 3, and the *I* or *F* model. Each individual is represented as a vertical bar partitioned into K segments, whose length is proportional to the estimated membership in the K clusters. The horizontal black lines indicate values of individual proportion of membership $q_i = 0.20$ or 0.80.

Admixture analyses in the Italian wildcats

Admixture analyses in the Italian wildcats were performed using STRUCTURE with $K = 3$, comparing the results obtained with the two marker sets and the *F* or *I* models. We used the following benchmarks to evaluate the performances of admixture analyses: (i) all sampled domestic cats are true domestic and have no recent ancestry in any wildcat population; (ii) the known hybrids obtained in captivity are F_1 or backcrosses with wildcats. Therefore, admixture analyses should correctly identify all domestic and all known hybrids, at a given threshold probability, using 21 or 27 loci, and the *F* or *I* models.

A threshold probability $q_i = 0.90$ would exclude 21 or 17 domestic cats (using the *I* model with 21 or 27 loci, respectively), and 6 or 4 domestic cats (using the *F* model with 21 or 27 loci, respectively) from the domestic cat cluster (Fig. 4). Hence, we used a lower threshold probability $q_i = 0.80$ to infer the ancestry of individual cats. The 21 linked loci misclassified 8 (using the *I* model) and 3 (using the *F* model) domestic cats, correctly recognized 5 (*I* model) and 4 (*F* model) of the known hybrids, and identified 8 (*I* model) or 7 (*F* model) admixed Italian wildcats. The total set of 27 loci misclassified 2 (*I* model) or no (*F* model) domestic cats, correctly recognized 5 hybrids (HY62, which plots within the domestic cat cluster in the CA, was not detected; see Figs 2 and 5), and 5 admixed Italian wildcats, independently on the model. In summary, 27 loci and the *F* model performed better. In these conditions, 5 Italian wildcats were inferred to be admixed (i.e. Fsi68, Fsi228, Fsi283, Fsi284 and Fsi580). Sample Fsi284 was previously found admixed, while Fsi68, Fsi228 and Fsi283 were previously classified as 'wild' using 12 unlinked loci (Randi *et al*. 2001). Sample Fsi580 showed some morphological signs of hybridization (B. Ragni, personal communication.). Cats Fsi68 and Fsi283 were morphologically classified as 'wildcats'.

A ternary plot obtained with STRUCTURE using $K = 3$, 27 loci and the *F* model (Fig. 5), showed a sharp distinction between the individual q_i values of the domestic cats (joining cluster I with $q_I > 0.80$), and those of most of the Italian wildcats (joining cluster II with $q_{II} > 0.80$), with the exception of samples Fsi228, Fsi283, Fsi284 and Fsi580, which where intermediate between the domestic and Italian wild cat clusters. Sample Fsi68 plotted close to $q_{II} = 0.80$. The identification of the known hybrids was straightforward,

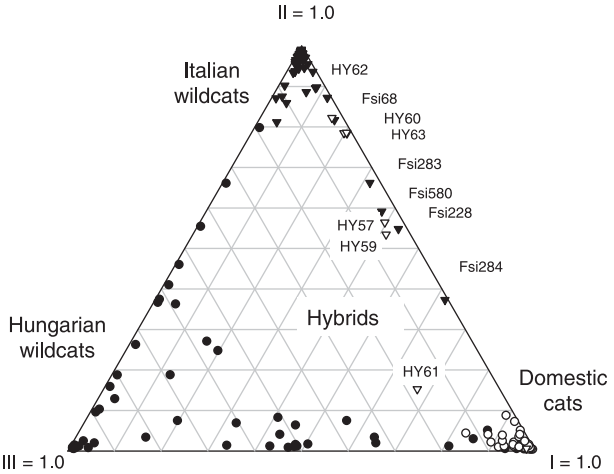

Fig. 5 Ternary plot of individual proportions of membership computed by STRUCTURE with $K = 3$, the *F* model and 27 microsatellite loci. Samples plotting at the apices have proportion of membership close to $q_i = 1.0$ to cluster I (domestic cats), II or III (wildcats). Shaded areas indicate the threshold $q_i = 0.80$. Known hybrids (white triangles) and admixed Italian wildcats (black triangles) are labelled.

because samples HY57, HY59, HY60, HY61 and HY63 plotted intermediate between the domestic and Italian wild cat clusters, while HY62 plotted within the Italian wildcats close to the $q_{II} = 0.80$ value (see also Fig. 2).

Admixture analyses of Hungarian free-living cats

We used STRUCTURE with $K = 3$, 27 loci and the *F* model to analyse the ancestry of the free-living Hungarian cats. In these conditions, 30 Hungarian cats (46%) were assigned to clusters II and III (the wildcat clusters) with $q_{II+III} > 0.80$. These cats could be identified as wildcats (Fig. 5). Nineteen (29%) Hungarian cats were included into the domestic cat cluster I, while there were 16 (25%) samples intermediate between cluster III and the domestic cat cluster I, which could be genetically admixed.

The Hungarian cats were classified phenotypically before genetic analyses, and accordingly they were subdivided in 17 (26%) wildcats, 18 (28%; plus one uncertain) domestic cats, and 28 (45%; plus one uncertain) hybrids. Phenotypic and genetic identifications of domestic cats were fully concordant, while 13 phenotypic hybrids were assigned to the wildcat clusters using genetic data. Fourteen of the 16 genetic hybrids identified by STRUCTURE were also identified as hybrids morphologically (one was identified as a wildcat). However, 14 phenotypic hybrids were classified as wildcats using genetic data. Nevertheless, the Spearman rank correlation between the morphological and genetic assignments was highly significant: $Z = 6.70$ ($P < 0.001$).

Estimating the origin of chromosomal segments and admixture times in Italian and Hungarian cats

The linkage model in STRUCTURE 2.1 allows inference of the ancestry of chromosomal chunks, making thus possible to identify the population of origin of each allele (Falush *et al*. 2003). A first analysis was designed to test the hypothesis that both M (maternal) and P (paternal) alleles derived from the wildcat population 1, or from the domestic cat population 2, that is, Ln $P(R)_1$ = (M1P1/M2P2). Positive values of this test support the assignment of the alleles to the wildcat gene pool. The distributions of Ln $P(R)_1$ in the Italian sample was bimodal, with modes at Ln $P(R)_1 = -10$ and 10 (Fig. 6a). There were 1594 (96.8%) tests that involved putative wildcats and that showed values of Ln $P(R)_1 > 0$, while only 53 (3.2%) tests evidenced values of Ln $P(R)_1 < 0$. Inspection of individual genotype assignments showed that all except five values of Ln $P(R)_1 < 0$ were attributed to Fsi68, Fsi228, Fsi283, Fsi284 and Fsi580, in agreement with their admixed ancestry (these cats were assigned to the wildcat cluster with $q < 0.80$; see also Fig. 2). The remaining five negative values were attributed to Fsi508, which showed a value of $q = 0.89$. None of the 1242 tests involving domestic cat samples showed values of Ln $P(R)_1 > 0$. The known hybrid cats showed a predominance of positive values of Ln $P(R)_1$ ($n = 104$) over negative values ($n = 58$), according to their origins. The distribution of Ln $P(R)_1$ values in the Hungarian free-living cats showed three modal values at Ln $P(R)_1 = -7$, 0 and 9, and the lower tail of the distribution overlapped the distribution of the domestic cats (Fig. 6b). There were 102 tests showing values of Ln $P(R)_1 = 0$, indicating that the assignments of chromosomal chunks to the wild or domestic cat gene pools were equally likely, and that were attributed to 16 samples which were already identified as admixed (i.e. they were assigned to the wildcat gene pool with $0.387 \leq q \leq 0.630$).

In a second analysis, we tested the hypothesis that both alleles at each locus came from a pure population (parental) against the hypothesis that one allele came from one population and the second allele came from the other population (recombinant), that is, Ln $P(R)_2$ = (M1P1 + M2P2)/(M1P2 + M2P1). The frequency distribution showed only positive values of Ln $P(R)_2$ in the domestic cats confirming their nonadmixed genetic composition (Fig. 6c, d). The distribution of Ln $P(R)_2$ values of the known hybrids overlapped the lower tail of the distribution of the Italian wildcats (Fig. 6c). There were 63 (3.8%) negative values of Ln $P(R)_2$ over 1647 tests in the Italian samples, due to cats Fsi68, Fsi228, Fsi284, Fsi508, Fsi575, Fsi576 and Fsi580; some of them already identified as admixed with proportion of membership to the wildcat cluster $0.366 \leq q \leq 0.950$. The frequency distribution of the admixed Hungarian sample was bimodal, with modes at Ln $P(R)_2 = 4$ and Ln $P(R)_2 = 0$ (Fig. 6d). There were 313

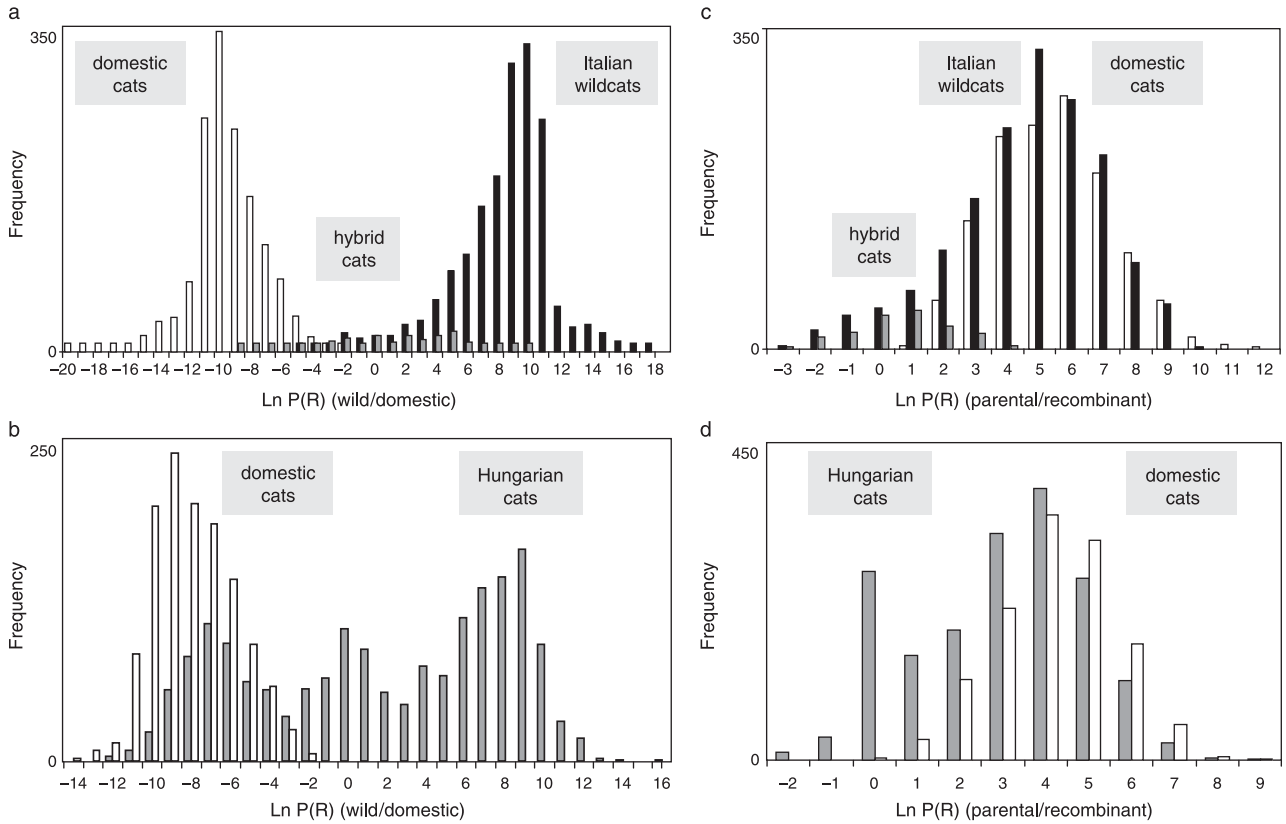

Fig. 6 Distributions of the population-of-origin assignments for the five microsatellite linkage groups in domestic, Italian wild, Hungarian free-living and known hybrid cats (see Results).

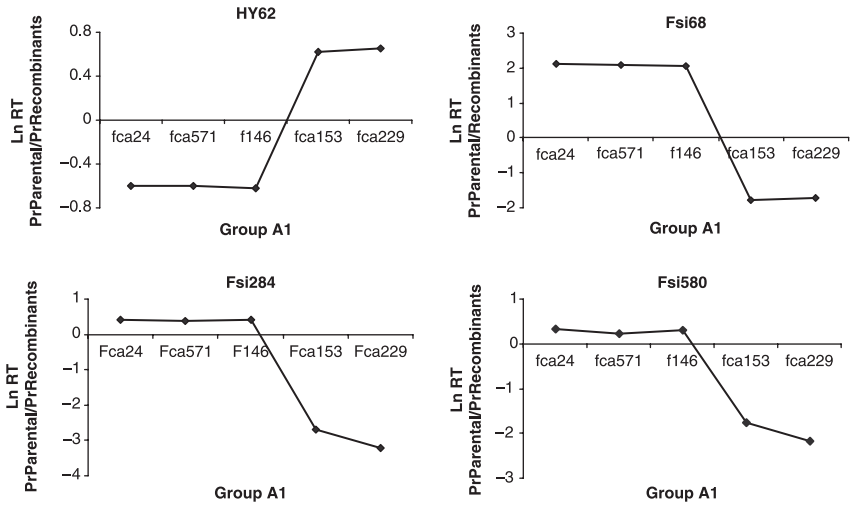

Fig. 7 Examples of inferred recombination events among microsatellite loci in linkage group A1, in known hybrid cat HY62 and in admixed Italian wildcats Fsi68, Fsi284 and Fsi580.

tests (17.8%) showing values of Ln $P(R)_2 \leq 0$ in 19 Hungarian cats, indicating admixture and including all the 16 cats which were already identified as admixed with $0.387 \leq q \leq 0.711$.

We detected many sharp changes in Ln P(R) within the microsatellite linkage groups of individual cats, which might indicate the position of putative recombination events. For instance, values of Ln $P(R)_1$ = (M1P1/M2P2) in linkage group A1 changed rapidly between loci F146 and Fca153, suggesting recombination between parental chromosomes in the two known hybrid cat HY62 and in Italian admixed cats Fsi68, Fsi284 and Fsi580 (Fig. 7). Similar signals of recombination events within linkage groups were

identified in the known hybrids, as well as in the admixed cats from Italy and Hungary.

The value of the parameter *r*, estimated as average of 25 repeated runs of STRUCTURE with $K = 3$, 27 loci, *F* model (map distances in cM), was $r = 0.15$ (SD = 0.13; SE = 0.03) for admixture between domestic and wild cats in Italy, which corresponds to 15 generations after the admixture. In contrast, average *r* for admixture between domestic and wild cats in Hungary was 3.75 (SD = 0.89; SE = 0.18), corresponding to 375 generations.

Discussion

Linked markers, correlated allele frequencies and assignment of admixed cats

In this study we show how a panel of closely linked microsatellite markers can be used to identify probabilistically wild × domestic cat hybrids, which were sampled from two populations with different admixture proportions. All domestic cats, five of the six known hybrids and five presumed hybrids among the Italian wildcats were identified by STRUCTURE with $K > 3$, using the total set of 27 linked and unlinked markers, and the *F* model. All those hybrids were also visually distinct in the CA plot (Fig. 2). Three Italian cats, which were previously morphologically described as wildcats, were, in contrast, identified as genetically admixed. Rank correlations between morphological and genetic assignments in Hungarian cats were highly significant ($P < 0.001$), although 13 cats that were morphologically identified as phenotypic hybrids were genetically assigned to the wildcat cluster. Uncertain definition of coat-colour diagnostic traits, or natural variation in external markers can result in incorrect morphological identifications. In contrast, the number of genetic markers used in this study may be still too low to correctly identify all the admixed individuals.

A drawback of the linkage model is that closely linked markers are nonindependent and less informative than the same number of independent markers. The number of independent linkage groups was small, and the addition of unlinked loci, as recommended by Falush *et al*. (2003), had the predictable consequence of improving the performances of admixture analyses. Our results suggest that a larger number of markers (linked and unlinked) could improve the identification of admixed cats.

Different admixture rates and times among wildcats in Italy and Hungary

Admixture analyses indicate that hybridization is not frequent in Italy, and that wild and domestic cats are reproductively isolated. In contrast, wild and domestic cats in Hungary are deeply introgressed, suggesting that hybridization is ongoing, or it has been frequent in the near past. The use of linked markers and the new linkage and *F* models implemented in STRUCTURE 2.1, confirm results obtained in the past using unlinked microsatellites to genotype cats sampled from the same populations in Italy and Hungary (Randi *et al*. 2001; Pierpaoli *et al*. 2003). However, the linked plus some unlinked markers used in this study showed a slight increase in the proportion of Italian wildcats which were identified as admixed. This result was expected because correlations among closely linked markers decay slowly, and the linkage model can identify more ancient admixture events. Simultaneously, the new *F* model in STRUCTURE 2.1, which allows for correlations in allele frequency among populations, performed better than the *I* model.

It is worth noting that standard estimators (i.e. F_{IS} and Weir's *R*) did not evidence any strong HWD and LD in the studied cat groups. LD was marginally higher, as predictable, in the Hungarian highly admixed cats than in the Italian wildcats, which behave as a random-mating population with low admixture rate and low frequency of hybridization with domestic cats. Low LD in the admixed cats in Hungary suggests that this population may not hybridize frequently, and that hybridization is perhaps more an historical than a current event. In fact, the posterior values of the parameter *r* estimated for admixture between domestic and wild cats in Hungary (average $r = 3.75$) were about 25 times larger than in Italy (average $r = 0.15$), revealing that admixture in Hungary might be traced back to 375 generations in the past.

These admixture times are certainly approximate, because real admixture processes may be much more complex than the demographic model implemented in STRUCTURE (as well as in other methods; Chikhi *et al*. 2001; Excoffier *et al*. 2005), which assumes a single admixture event between two ancestral populations t_2 generations ago. Admixture in cats likely involves repeated or continuous hybridization events. The Italian and Hungarian cats clustered separately both in the CA plot (Fig. 2) and in the Bayesian analyses (Fig. 5), indicating geographical population structuring. Distinct phylogeographical and historical components could have shaped the structure of wild and, perhaps, also domestic cats in Hungary, thus making admixtures more complex than a simple mixing of indistinct wild and domestic parental populations. A reconstruction of the phylogeography of wildcats in the Palaearctic, which is still lacking, would probably aid to develop a better description of admixtures. Therefore, the value of *r* should be interpreted with caution. However, despite simplifications, the linkage model might pick up correctly the main historical components of the admixture process, showing that hybridization is more widespread and ancient among wildcats in Hungary than in Italy.

The five Italian wildcats that resulted admixed in most of the analyses were sampled from San Severino (Regione Marche; Fsi580), Enna (Sicily; Fsi228), Grosseto (Tuscany Maremma; Fsi68, Fsi283 and Fsi284; locations are indicated by arrows in Fig. 1), thus confirming results by Randi *et al*. (2001) that hybridization in Italy is more frequent at geographical and ecological edges of the wildcat distribution. Regione Marche and Tuscany Maremma are, respectively, the northern and western borders of the wildcat range in Italy. The Tuscany coastal area, with Mediterranean ecosystems, presents a higher density of free-ranging domestic cats than in the forested Apennine core areas of the wildcat distribution (Ragni *et al*. 1994). Cats sampled from the northeast of Hungary showed a prevalence of hybrids in the areas between Babat, in the north, and Dèvavànya, in the south (Fig. 1). The northeast regions of Hungary are characterized by widespread urbanization, agriculture and mosaics of forests and human settlements, creating an environment where encounters between free-ranging domestic and wild-living cats are facilitated.

Detecting recombination events and perspectives for association analysis in wild populations

Evidences that alleles derived from one or the other parental population (i.e. domestic or wild cats) were assessed using ratio tests of the probability of ancestry of chromosomal chunks (Seldin *et al*. 2004). The shapes of the frequency distributions of Ln P(R) values were correspondent to expectations based on the genetic composition of domestic, Italian wild and Hungarian admixed cats. Known hybrids and admixed individuals were also identified, congruent with individual q_i values. Moreover, a number of putative recombination events, marked by sharp changes of the Ln P(R) values between adjacent loci, were identified only in the known hybrids or in the admixed cats. The genotypes in our data set were unphased, thus conveying less information on the population of origin of the alleles and on the admixture time.

These results show that chromosomal blocks (Stephens *et al*. 1994) can be identified, and admixture LD mapping analyses (Briscoe *et al*. 1994) can be designed also in natural populations, such as it was done in humans or in model organisms (Briscoe *et al*. 1994). Admixture LD analyses can be designed to detect associations between molecular markers and candidate genes or phenotypic traits also in populations which cannot be manipulated for QTL experiments. In perspective, it should be possible to identify chromosomal regions bearing functional genes which are maintained in LD with flanking neutral markers by natural selection (i.e. local adaptations), or as a by-product of artificial selection (i.e. domestication genes). This strategy could lead to identify adaptive genes (fitness genes, disease resistance genes) in natural populations, if high-density markers maps could be developed (Wu & Zeng 2001).

Conclusions

Hybridization and conservation of wildcat populations

The wildcat is listed in the Annex IV of the European Community Species and Habitat Directive (Bern Convention), as well as legally protected in most European countries (Stahl & Artois 1994). However, the main threatening factors (illegal persecution, accidental killings, habitat loss and fragmentation) still persist. Habitat modifications and animal translocations increased the rates of hybridization and introgression, raising conservation concerns especially when hybridization occurs between protected species and their domesticated forms (Allendorf *et al*. 2001; Daniels & Corbett 2003).

Hybridization is widespread in Hungary where from about 25% (using microsatellites) to 45% (using only morphological identifications) free-living cats might be admixed. It is not clear why domestic and wild cats do hybridize more frequently in some areas than elsewhere in Europe (Beaumont *et al*. 2001; Pierpaoli *et al*. 2003). A number of historical factors (i.e. strong local decline of wildcat populations due to deforestation and direct persecution), or landscape structure in agro-forest ecosystems (i.e. mosaics of small forest patches embedded in agricultural areas) could differentially affect the outcomes of reproductive interaction between free-ranging domestic and wild cats (Beaumont *et al*. 2001; Daniels *et al*. 2001; Randi *et al*. 2001). Our data for instance suggest that hybridization may be more frequent in marginal areas in Italy, where the density of wildcats is low, or that were recently colonized by wildcats, but where the density of free-ranging domestic cats is overwhelming.

While further research on the ecology of feral and hybrid cats would certainly contribute to design sound long-term wildcat conservation strategy, the available genetic and morphological data have documented that hybridization is a main threat to genetic integrity of wildcat populations in Europe. Conservation measures aimed to prevent or reduce hybridization should be directed to control the density of free-ranging domestic cats. In this perspective, improving the diagnostic power of both molecular and morphological markers would allow for a better monitoring of hybridization.

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